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Immunology

Site-specific covalent modification of monoclonal antibodies: *In vitro* and *in vivo* evaluations

(antibody modification/glycoprotein modification/tumor localization/nuclear imaging/immunoscintigraphy)

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ABSTRACT **A strategy for covalent modification of monoclonal antibodies utilizing the oxidized oligosaccharide moieties on the molecule was evaluated and compared to more conventional methods. As judged by quantitative *in vitro* measurements, a monoclonal antibody conjugate prepared via the oligosaccharides retained the homogeneous antigen binding property and affinity of the unmodified antibody. In contrast, conjugates of the same antibody, modified to the same degree on either lysines or aspartic and glutamic acid side chains, were heterogeneous in their antigen binding and had lowered affinity. *In vivo* biodistribution and nuclear-imaging experiments were also performed with a second monoclonal antibody and a tumor xenograft model. Antibodies modified on the oligosaccharides with either (*i*) a peptide labeled with iodine-125 or (*ii*) a diethylenetriaminepentaacetic acid chelate with indium-111 localize into target tumors more efficiently than the same antibody radiolabeled on either tyrosines or lysines. These *in vivo* results, when compared to those reported in the literature for conventionally modified antibodies, suggest that oligosaccharide modification of monoclonal antibodies is a preferred method of preparing conjugates.**

The development of monoclonal antibody technology (1) has given new emphasis to the potential use of antibodies for targeting various substances to tumors *in vivo*. To this end, monoclonal antibodies have been developed for a number of human neoplasms, some of which have already been evaluated in patients (2–5). For *in vivo* diagnostic applications, antibodies typically are labeled with γ-emitting isotopes for use in immunoscintigraphy. Labeling is achieved by coupling iodine-131 or iodine-123 to tyrosines (6–14), by coupling indium-111 to chelators that are coupled to lysines (12, 15–19), or by passively adsorbing technetium-99m (20). For therapeutic applications, antibodies are sometimes used as unmodified proteins (2, 5), but more commonly they are attached to isotopes (4, 21, 22), toxins (23), toxin fragments (24, 25), or any one of a number of cytotoxic drugs (9, 26–28) in attempts to target these agents to tumors. These latter modifications all involve covalent attachment to tyrosines, ϵ-amino side chains of lysines, carboxyl side chains of aspartic and glutamic acids, or sulfhydryl groups generated by reduction of cystines. Although each of these applications has shown promise, none, as yet, has reached the point of proven clinical utility.

Since so many of the diagnostic and therapeutic applications envisioned for monoclonal antibodies require coupling of antibodies to other substances, there is a need for methods of covalent modification that can be used on a broad spectrum of different antibodies with minimal effect on antigen binding properties. This paper will present a strategy for site-specific covalent modification of antibodies based upon attachment through the oligosaccharide moiety of the molecule. Because of the restricted localization of the glycosylation sites on immunoglobulins, such an approach offers the potential advantage of modification of the antibodies at a site distal to the antigen combining site (29). Data will be presented that first compares the effect of various techniques of monoclonal antibody modification on the antigen-binding homogeneity and affinity of the conjugate. Second, the comparative *in vivo* biodistribution and tumor localization of additional radiolabeled monoclonal antibodies will be presented using nude mice bearing subcutaneous tumor xenografts.

MATERIALS AND METHODS

***In Vitro* Binding.** The mouse monoclonal anti-phosphocholine IgM, HPCM2 (30), was grown in BALB/c mice and purified from ascites by ammonium sulfate precipitation, followed by affinity chromatography (31). The following conjugates of HPCM2 were prepared.

(*i*) For oligosaccharide attachments, the oligosaccharides moieties were oxidized to aldehydes by incubation in the dark with 10–30 mM $NaIO_4$ in phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate) at pH 6.0 on ice for 1 hr. After passage through a Sephadex G-25 column equilibrated with phosphate-buffered saline at pH 6.0, oxidized antibody was incubated with a 270-fold molar excess of 1,6-diaminohexylethylenediaminedi-*o*-hydroxyphenylacetic acid (1,6-diaminohexyl-EDDHA)[§] for 1 hr at room temperature. Sodium cyanoborohydride (Aldrich) was added to a final concentration of 10 mM, and the solution was incubated for an additional 4 hr and then dialyzed at 4°C versus several changes of phosphate-buffered saline.

(*ii*) For aspartic/glutamic acid attachments, the antibody was incubated in 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma) at pH 5.9 for 2 hr at room temperature. The 1,6-diaminohexyl-EDDHA was added to a final concentration of 10 mM, and the solution was incubated at room temperature for an additional 2 hr. Ten microliters of 1 M ethanolamine was added, and, after 1 hr the reaction mixture was dialyzed versus phosphate-buffered saline.

(*iii*) For lysine attachments, the scheme for aspartic/glutamic acid modification was used except that EDDHA (5 mM; Sigma) replaced the 1,6-diaminohexyl-EDDHA.

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Abbreviations: EDDHA, ethylenediamine-di-*o*-hydroxyphenylacetic acid; DTPA, diethylenetriaminepentaacetic acid; MHC, major histocompatibility complex; T/B, tissue-to-blood ratio; RES, reticuloendothelial system.

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§The synthesis of these compounds will be published elsewhere.

For all preparations, the average number of groups attached was five, as determined by the formation of the fluorescent EDDHA chelate with terbium (32).

Affinities of the native anti-phosphocholine antibody and the conjugates for the ligand *N*-(2,4-dinitrophenyl)-*p*-aminophenylphosphocholine were measured by the technique of fluorescence quenching (33) as described elsewhere (34).

***In Vivo* Biodistribution.** The monoclonal antibody (R9.75) used for the *in vivo* biodistribution experiments is a rat IgG2c specific for a class I major histocompatibility complex (MHC) antigen of BN rats (35). Ascites was produced in nude mice, and the antibody was purified by gradient elution from a protein A-Sepharose column (Pharmacia) (36).

The control antibody was a mouse monoclonal IgG2b, designated KE2, specific for a human HLA determinant (a gift from Roger Kennett, University of Pennsylvania).

Purity of all antibody preparations was confirmed by $NaDodSO_4$/polyacrylamide gel electrophoresis (37).

^{125}I-labeled conjugates. R9.75 was labeled on endogenous tyrosines with $Na^{125}I$ (Amersham) using the chloramine-T method (38). Alternatively, the synthetic pentapeptide glycyltyrosylglycylglycylarginine, prepared by standard solid-phase synthetic methods (39), was radiolabeled at the tyrosine (38) with $Na^{125}I$ before coupling to the oxidized oligosaccharides of R9.75 as described.

^{111}In-labeled diethylenetriaminepentaacetic acid (DTPA)-antibody conjugates. In brief, these conjugates were prepared in two ways.

(*i*) Oligosaccharide attachment. R9.75 or KE2 oligosaccharides were oxidized as described, and then the modified antibodies were incubated with either glycyltyrosyllysyl-DTPA or *p*-aminoaniline-DTPA. Incubation conditions were as described above, except that a 1000-fold molar excess of chelator was used. Two to five hundred micrograms of these antibodies in phosphate-buffered saline were radiolabeled with ^{111}In (New England Nuclear). Radiolabeled antibodies were separated from free ^{111}In by elution through a Sepharose G-50 column. The specific activities ranged from 1 to 5 μCi/μg (1 Ci = 37 GBq).

(*ii*) Lysine attachment. R9.75 was coupled to DTPA and incubated with ^{111}In as described (17).

Tumor models consisted of female nude mice (NIH Swiss–Webster, Taconic Farms, Germantown, NY) that had been injected subcutaneously in the left flank with 5×10^6 BN lymphoma cells. When tumors were 3–5 mm in diameter, radiolabeled antibodies were injected i.v. into tumor-bearing and control nude mice either through the tail vein or the medial canthus. Animals injected with the ^{125}I-labeled conjugates were sacrificed and dissected 24 hr after injection, and organs were weighed and counted. Animals that received the ^{111}In-labeled conjugates were imaged without background subtraction at 24, 48, and 74 hr after injection by using a General Electric Maxi Camera 37 equipped with an ADAC computer system. Alternatively, animals were sacrificed and dissected 48 hr after injection to obtain quantitative biodistribution data.

RESULTS

***In Vitro* Binding Experiments.** The first part of the study was designed as a quantitative test of the comparative effects of oligosaccharide, lysine, or aspartic/glutamic acid coupling on the antigen-binding properties of a monoclonal antibody. Conjugates of the phosphocholine-binding mouse monoclonal IgM antibody, HPCM2, were prepared, and affinity and homogeneity were measured by fluorescence quenching (33). The binding data were analyzed by using the Sips distribution function (40) and are shown in Fig. 1. As described elsewhere

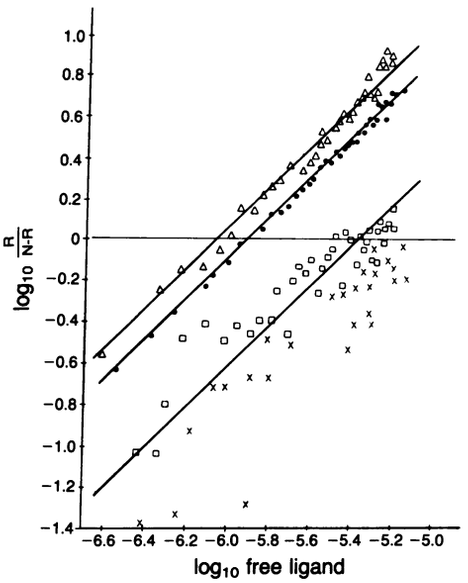

FIG. 1. Sips plot of fluorescence quenching data for HPCM2 (30). R, fractional saturation of binding sites; N, antibody valence; ●, unmodified antibody ($K = 8 \times 10^5$ M^{-1}, correlation coefficient = 0.996); △, oligosaccharide-modified antibody ($K = 1 \times 10^6$ M^{-1}, correlation coefficient = 0.994); □, aspartic/glutamic acid-modified antibody ($K = 1 \times 10^5$ M^{-1}, correlation coefficient = 0.926); ×, lysine-modified antibody.

(34), this analysis assumes that each sample is composed of a homogeneous population of antigen-binding molecules. When this is the case, the data will fit a line with a slope of 1. In fact, the data for the unmodified monoclonal antibody and the oligosaccharide conjugate fit a line of such slope. In contrast, data for the other samples deviate significantly from such a line, which is evidence that these conjugates are composed of a mixture of antigen-binding molecules with different affinities. Furthermore, this analysis yields a value for the association constant of the antibody preparations that derives solely from the active antigen binding proportion in each preparation (34). Compared to the unmodified antibody, the conjugates coupled directly on amino acid side chains had significantly lower affinities, while the oligosaccharide-coupled conjugate did not (Fig. 1).

***In Vivo* Biodistribution.** The *in vivo* localization of different conjugates of a monoclonal antibody, R9.75, was evaluated by injection into nude mice bearing BN lymphoma xenografts on their left flank. Normal nude mice were used as controls. Animals were dissected 24 or 48 hr after injection, and the organs were weighed and counted. The percentage of total injected dose per gram of tissue and tissue-to-blood ratios (T/B) were then calculated.

The first comparison was between R9.75 that was ^{125}I-labeled by direct iodination of tyrosines and R9.75 that was coupled through the oligosaccharides to a radioiodinated peptide. The preparations had equivalent *in vitro* cell-binding activities (Table 1). The biodistribution data from dissection and organ counting of animals 24 hr after injection are shown in Table 1. There was little nonspecific uptake of either conjugate into any organ of the control mice, but a comparison of the percentage of the total injected dose per organ in tumor-bearing animals demonstrates greater tumor localization in animals injected with the oligosaccharide-linked conjugate compared to the directly iodinated antibody.

The T/B ratios show that the directly labeled antibody did not localize well to the tumor at this time point.

The next comparison involved similar evaluation of the *in vivo* behavior of ^{111}In-labeled R9.75. The first experiment was designed to compare standard modification chemistry

Table 1. ^{125}I-labeled antibody biodistribution 24 hr after injection

	Direct label				Oligosaccharide label			
	Tumor xenograft		Control		Tumor xenograft		Control	
Tissue	%ID per g	T/B	%ID per g	T/B	%ID per g	T/B	%ID per g	T/B
Tumor	2.5 ± 1.1	1	—	—	46 ± 1.1	5.5	—	—
Liver	0.6 ± 0.2	0.2	3	0.2	13 ± 0.8	2.4	18.2	0.5
Lung	2.0 ± 0.8	0.8	7.5	0.5	6.7 ± 0.1	1.1	20.2	0.6
Spleen	0.8 ± 0.3	0.3	3.3	0.3	8 ± 1.2	1.9	14.1	0.4
Kidney	0.8 ± 0.3	0.4	4.7	0.1	7.7 ± 0.1	1.5	17.2	0.5
Blood	2.4 ± 0.9	1	15.3	1	7.6 ± 0.6	1	35.6	1

In vitro cell binding activities were: direct label = 89%, oligosaccharide label = 85%. The percentage of total injected dose (%ID) retained at 24 hr in the tissues examined was: direct label = 19.3%, oligosaccharide label = 83%; %ID per g is %ID per g of tissue. T/B = cpm/g of tissue ÷ cpm/g of blood. Values are means ± SEM.

(17), involving attachment of DTPA to lysines, with oligosaccharide modification by the method described above. The specific activities achieved after chelation to ^{111}In were the same for both conjugates. BN tumor-bearing animals were imaged without background subtraction 24 hr after injection. Fig. 2 illustrates the results of this comparison. Fig. 2*A* shows the image obtained 24 hr after injection of the ^{111}In-labeled oligosaccharide-modified antibody conjugate. Fig. 2*B* is a photograph showing the typical size of the xenograft at this time. Fig. 2*C* is the image seen with the nonselectively modified antibody. The oligosaccharide-modified antibody localized well into the tumor, while the nonselectively labeled conjugate localized mainly to the liver region.

^{111}In-labeled oligosaccharide-modified R9.75 conjugates were further evaluated by imaging tumor-bearing mice 24, 48, and 72 hr after injection (Fig. 3 *A–C*). Mice imaged at all these time points showed clear tumor localization. A number of control experiments were also performed. Fig. 3*D* is a typical 24-hr image of a normal mouse injected with this antibody conjugate, in which no discrete tissue localization is observed. Fig. 3*E* is the 24-hr image obtained after the injection of a similarly labeled control antibody into a BN tumor-bearing mouse and Fig. 3*F* is the image obtained 24 hr after injection of the labeled R9.75 antibody into a mouse bearing a control human lymphoma xenograft. Again, no tumor localization is observed with these controls.

Quantitative analysis of the *in vivo* biodistribution of oligosaccharide-modified ^{111}In-labeled antibodies was undertaken by organ dissection studies (Table 2). Data for control mice illustrate that the cpm are distributed throughout the

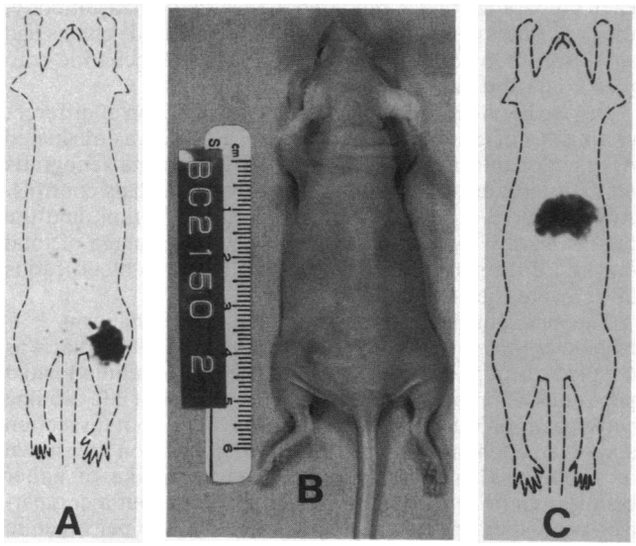

FIG. 2. BN tumor-bearing animals. (*A*) Twenty-four-hour image recorded after injection of the ^{111}In-labeled oligosaccharide-modified R9.75 conjugate. (*B*) Photograph showing the size and location of the tumor at the time of a 24-hr image. (*C*) Twenty-four-hour image recorded after injection of the lysine-coupled R9.75 conjugate. Animals were imaged ventrally, so the position of the tumors in *A* and *C* are reversed compared to *B*. Animal outlines (*A* and *C*) show approximate size and orientation of animals in relation to the images.

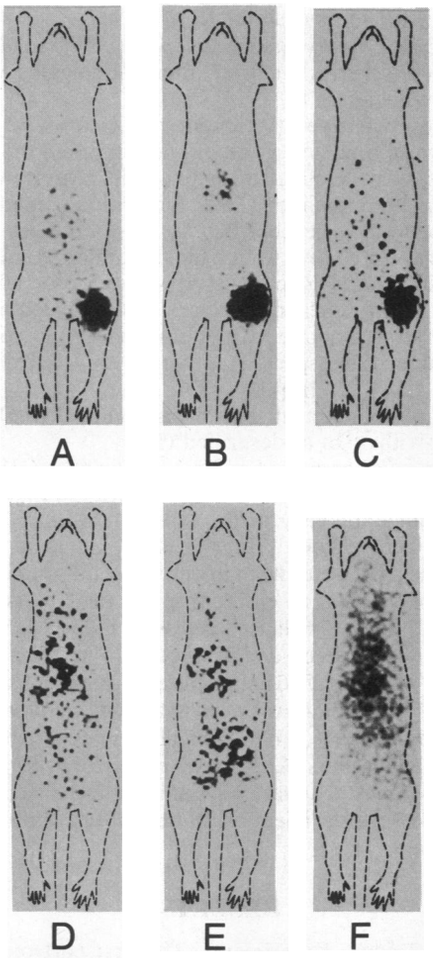

FIG. 3. BN tumor images and controls. (*A–C*) Images recorded 24, 48, and 72 hr after injection of the ^{111}In-labeled oligosaccharide-modified R9.75 conjugate into BN tumor-bearing nude mice. (*D*) The same conjugate 72 hr after injection into a normal mouse. (*E*) Control ^{111}In-labeled oligosaccharide-modified KE2 conjugate 72 hr after injection into a BN tumor-bearing nude mouse. (*F*) The ^{111}In-labeled oligosaccharide-modified R9.75 conjugate 24 hr after injection into a control mouse bearing a nonimmunoreactive human xenograft.

Table 2. ^{111}In-labeled oligosaccharide-modified antibody biodistribution 48 hr after injection

Tissue	T/B ratio		%ID per g of tissue	
	Control*	Tumor†	Control*	Tumor†
Blood	1	1	9.8 ± 1.2	1.4 ± 0.4
Lung	0.6 ± 0.1	1.8 ± 0.1	8.3 ± 0.6	1.9 ± 0.2
Spleen	0.7 ± 0.1	4.0 ± 0.6	5.7 ± 0.9	3.2 ± 0.2
Liver	0.9 ± 0.2	4.0 ± 0.6	6.0 ± 0.4	3.3 ± 0.2
Kidney‡	1.5 ± 0.3	6.8 ± 0.7	10.4 ± 1.2	5.6 ± 0.3
Skeletal muscle	0.2 ± 0.1	0.7 ± 0.1	1.3 ± 0.2	0.7 ± 0.1
Tumor	—	22.9 ± 1.7	—	22.3 ± 1.9

The percentage of total injected dose (%ID) retained at 48 hr was: tumor bearing mice = 86%, control mice = 92%. Values are means ± SEM.
*n = 13.
†n = 44.
‡Average of both kidneys.

various organs with a distribution that appears to reflect the relative cardiac output to these tissues. The data for the tumor-bearing animals support the extent of localization implied in Figs. 2 and 3.

DISCUSSION

This study was undertaken in order to compare, both *in vitro* and *in vivo*, site-specific covalent modification of monoclonal antibodies via their oligosaccharides with more conventional methods involving tyrosine, lysine, and aspartic/glutamic acid attachment. Chemical or enzymic oxidation of antibody oligosaccharides to aldehydes yields unique functional groups on the molecule, which can selectively react with compounds containing, for example, amines, hydrazines, hydrazides, and semicarbazides (41–43). Since the sites of attachment of oligosaccharides to antibodies are specific and distal to the antibody-combining site (29), selective coupling to aldehydes should yield more uniform antibody conjugates with unimpaired antigen-binding characteristics. In contrast, a common feature of covalent modification at tyrosines, lysines, or aspartic/glutamic acids is the lack of selectivity inherent in the methods used. Lack of selectivity in this context refers to the inability to control the coupling to a particular reactive amino acid at a specific location on the molecule. This leads to conjugates with a distribution of attachment sites, some of which may be located at or near the antibody-combining site. The result could be weakened or lost antigen-binding activity for some proportion of the antibody conjugates, depending upon the degree to which the architecture of the combining site is perturbed, or access blocked, by covalent attachments. The *in vitro* binding data in Fig. 1 support the inferred advantage of covalent modification of monoclonal antibodies at the oligosaccharides over conventional methods; namely, that such conjugates retain the homogeneous antigen-binding properties and affinity of the unmodified protein. In contrast, conjugates of the same antibody prepared by coupling to lysines or to aspartic/glutamic acid residues have both reduced affinity and heterogeneous antigen-binding properties.

In vivo, the utility of monoclonal antibodies depends in part upon their homogeneous antigen-binding specificity. It was important, therefore, to determine if modification at the oligosaccharide yields covalent conjugates that express their immunological specificity better than conventional conjugates *in vivo*. The initial comparison involved biodistribution experiments in normal and tumor xenograft-bearing nude mice by using a monoclonal antibody modified with ^{125}I either on endogenous tyrosines or coupled to a tyrosine-containing peptide, which was then site-specifically attached to oxidized oligosaccharides. After 24 hr (Table 1) the conjugate modified with ^{125}I on the oligosaccharides localized in the tumor with a 18-fold greater efficiency than the corresponding conjugate modified nonselectively on tyrosines. This occurred in spite of comparable *in vitro* cell binding activities for the two preparations. This suggests that *in vivo* localization to small subcutaneous tumors is a more stringent test of the immunological activity of an antibody conjugate than *in vitro* cell binding experiments. Both normal and tumor-bearing mice injected with the directly labeled antibody had poor retention of radioactivity after 24 hr. This is attributed to dehalogenation in the liver followed by loss of the ^{125}I via excretion. Since the animals' thyroids were blocked by a large excess of free iodine in their drinking water, uptake of ^{125}I at this site could not be evaluated. Strikingly, >80% of the conjugate [^{125}I]iodinated on the oligosaccharides was retained by the animals that received this preparation, perhaps suggesting that positioning the radiolabel on the oligosaccharides may shield it from dehalogenation.

The biodistribution and tumor localization data *in vivo* suggest that antibodies radiolabeled via their oligosaccharides might represent improved immunoscintigraphy reagents. It was then desirable to see whether ^{111}In-labeled oligosaccharide-modified antibody conjugates would lead to the same extent of hepatic uptake reported by others for nonselective ^{111}In conjugates (12, 15–19). The monoclonal antibody R9.75 was modified either by coupling the chelator DTPA nonselectively to lysines or by attaching suitable DTPA derivatives to oxidized oligosaccharides.

Several points are clear from the images. Localization of the ^{111}In-labeled oligosaccharide-modified antibody conjugate to the tumor occurred during this 24-hr interval, while the nonselective ^{111}In conjugate localized in the liver region. As before, this may be due to more rapid uptake of the nonselective conjugate by the RES. Although perhaps an extreme example, this illustrates the problems seen in the literature for ^{111}In-labeled antibody conjugates. Speculation as to the reasons for the observed hepatic uptake of nonselective ^{111}In-labeled antibody conjugates are numerous and can be divided into two categories: tumor-associated localization and nontumor-associated localization. Tumor-associated hepatic localization would be caused by specific antibody–antigen interactions and could be due to any or all of the following: RES uptake of immune complexes; the binding of antibody to antigen which, after shedding, was transiently expressed on the surface of cells of the RES; or the presence of foci of metastatic tumor growth in the liver. The absence of hepatic uptake of the ^{111}In-labeled oligosaccharide-modified antibody demonstrates that, for this model, there is little antigen that is not associated at the tumor site.

Nontumor-associated hepatic localization could be caused by antibody–Fc receptor interactions, the accumulation of denatured or aggregated antibody, or instability of the ^{111}In chelate. Localization into the liver or other tissues could also be the result of expression of the antigen by normal cells. These nontumor-associated events can be evaluated by biodistribution analyses of the antibody in normal animals. The image in Fig. 3*D* and the data in Table 2 demonstrate that the ^{111}In-labeled oligosaccharide-modified antibody conjugate is stable *in vivo* and does not show preferential localization into any particular normal organ or tissue. In fact, these data suggest that site-specific conjugates persist in circulation. Taken together, it appears that antibodies modified on the oligosaccharides are stable conjugates able to express immunological specificity well *in vivo*. For this xenograft model, then, any secondary localization in the regional lymph nodes or liver would be suspected as possible sites of metastatic BN tumor growth. Confirmed metastases have been detected by imaging in this model and will be the subject of a later publication. The observed hepatic uptake of

the nonselective conjugate was most probably the result of the modification chemistry. Lysine modification appears to have altered the antibody in such a way that it was taken up by the RES system. It seems unlikely that this could be due to chelate instability, since DTPA was used as the chelator for both conjugates.

The images from tumor-bearing animals obtained at various times after injection (Fig. 3 *A–C*) show that the ^{111}In-labeled conjugate remains in the tumor over a period of several days. The localization within 24 hr is more rapid than the rates of uptake in several reports using intact antibody (44, 45). The controls (Fig. 3 *D* and *E*) show that the localization is not due to some nonimmunological property of the tumor or the antibody: a different monoclonal antibody did not localize to the BN tumor xenograft, and the R9.75 antibody did not localize to a different tumor.

The qualitative impressions inferred from the images are quantitatively confirmed by dissection and assay of organs for radioactivity. The data are expressed in Table 2 in two different ways in order to make the point that no single parameter adequately represents the results. Expressed as the percentage of total injected dose per gram of tissue, small tumors would have values that could exceed 100%, although the absolute localization might actually be low. T/B ratios are useful, as they can shed light on what might otherwise appear to be selective tissue localization. Highly vascular organs such as lung, liver, spleen, and kidney can appear to be sites of localization, even in control animals, when organ counting data are expressed in other ways.

Taken together, these data suggest that oligosaccharide modification of monoclonal antibodies is a preferred method for preparing conjugates with particular utility *in vivo*. While antibodies with the appropriate immunologic specificity are a prerequisite for their successful use in diagnosis and therapy, this study suggests that selection of the method of covalent modification can also be an important consideration.

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